

Cryo-EM as a tool to study bacterial efflux systems and the membrane proteome

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Abstract

Antibiotic resistance is an emerging threat to global health. Current treatment regimens for these types of bacterial infections are becoming increasingly inadequate. Thus, new innovative technologies are needed to help identify and characterize novel drugs and drug targets which are critical in order to combat multidrug-resistant bacterial strains. Bacterial efflux systems have emerged as an attractive target for drug design, as blocking their export function significantly increases the potency of administered antibiotics. However, in order to develop potent and tolerable efflux pump inhibitors with high efficacy, detailed structural information is required for both the apo- and substrate-bound forms of these membrane proteins. The emergence of cryo-electron microscopy (cryo-EM) has greatly advanced the field of membrane protein structural biology. It has significantly enhanced the ability to solve large multi-protein complexes as well as extract meaningful data from a heterogeneous sample, such as identification of several assembly states of the bacterial ribosome, from a single data set. This technique can be expanded to solve the structures of substrate-bound efflux pumps and entire efflux systems from previously unusable membrane protein sample preparations. Subsequently, cryo-EM combined with other biophysical techniques has the potential to markedly advance the field of membrane protein structural biology. The ability to discern complete transport machineries, enzymatic signal transduction pathways, and other membrane-associated complexes will help us fully understand the complexities of the membrane proteome.

Keywords

Antibiotic resistance, RND-type transporters, cryo-electron microscopy

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Introduction

The widespread prevalence of multidrug-resistant (MDR) bacteria is an emerging threat to global health. Data released by the Centers for Disease Control and Prevention show that in the United States there are more than 2.8 million new cases of antibiotic-resistant infections each year¹ with considerably more worldwide². With the continual exposure of antibiotics given in standard treatment regimens, MDR, extensively drug-resistant (XDR), and totally drug-resistant (TDR) bacterial strains have emerged^{3,4}. These drug-resistant strains are especially problematic in hospital settings, where immunocompromised and other high-risk patients are extremely susceptible to bacterial infections. This is highlighted by a recent analysis of lungs harvested for use in transplant procedures; almost 60% of donor lung tissue had bacterial contamination, and about 5% of those contained MDR strains⁵. Whereas these pathogens currently can be eliminated with a tailored antibiotic treatment, it is well established that hospital-borne MDR bacteria such as *Acinetobacter baumannii*^{6–8}, *Staphylococcus aureus*^{9–11}, and *Escherichia coli*^{12–15} can develop resistance mutations through natural evolution, leading to the continual need to develop new antibiotic treatments. To help combat these superbugs, unique antibiotics and potent inhibitors of critical bacterial survival systems need to be developed. A common method for identification of new biocides is to conduct a blind screen for potential compounds from established chemical libraries and then test them in *in situ* bacterial systems^{16,17}. The binding capabilities of these compounds to their identified targets are often verified through docking studies and molecular dynamics simulations based upon previously determined protein structures¹⁸. Thus, the greater the number of high-resolution structures available for these modeling studies, the better the odds of identifying potent inhibitors and antibiotics through rational drug design, as opposed to random hits from a large chemical screen. Concurrent with this, the development of innovative biophysical and biochemical methods to produce high-quality structural data is critical to help identify and characterize unique targets for drug development.

Targeting efflux pumps to mitigate antibiotic resistance

Drug-resistant infections are the result, in part, of the alarming frequency in which bacteria can alter their genomes. Mutations¹⁹, as well as mobile genetic elements^{20,21}, are mechanisms that allow bacteria to acquire antibiotic resistance. These genetic changes often decrease the permeability of the outer membrane of the cell as well as increase the rate at which antibiotics are expelled²², and the latter is principally regulated through the function of bacterial efflux systems. These efflux systems are critical for survival by removing harmful agents detrimental to the cell²³. Additionally, in several infectious bacteria such as *Mycobacterium tuberculosis*, these exporters are also designed to transport lipid from the interior of the cell to the outer membrane for membrane biogenesis²⁴. Among various types of efflux systems, members of the resistance–nodulation–cell division (RND) superfamily are the most important in mediating antibiotic resistance in Gram-negative pathogens²⁵. The traditional architecture of an RND-type bacterial efflux system consists of an outer membrane

channel protein (OMP), a periplasmic membrane fusion protein (MFP), and an inner membrane efflux pump (IMP) (Figure 1A). The canonical structure of the RND-type IMP consists of 12 transmembrane helices that span the inner membrane of Gram-negative bacteria and several periplasmic domains

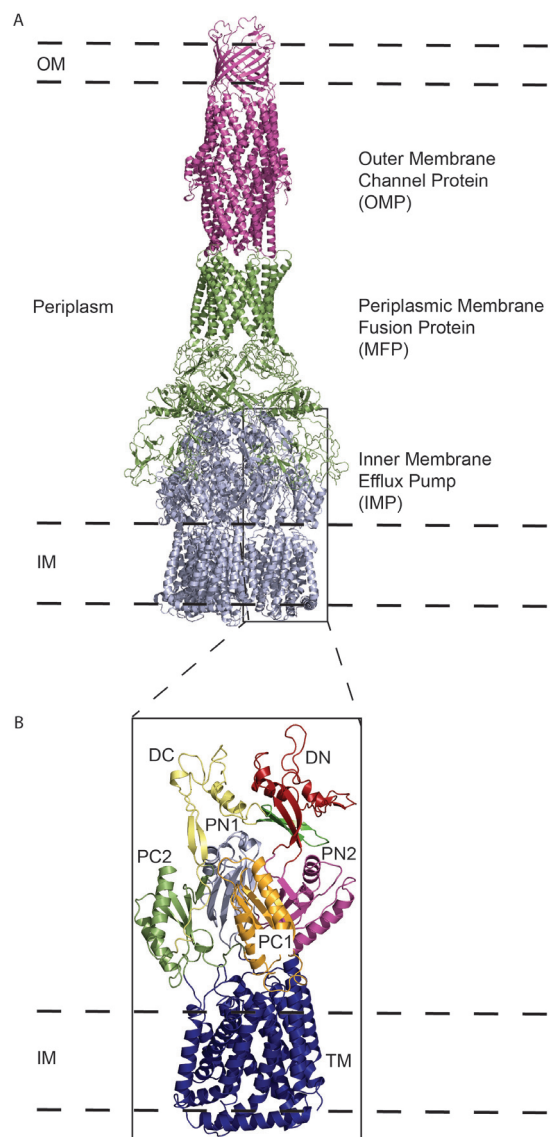


Figure 1. General structure of trimeric RND efflux systems. (A) The components of a tripartite efflux system (adapted from Protein Data Bank ID 5O66) visualized in side view with the inner membrane pump (IMP, blue), membrane fusion protein (MFP, green) and outer membrane protein (OMP, purple). Pictured is the IMP:MFP:OMP subunit ratio of 3:6:3, which is the most common assembly pattern. The outer membrane (OM) and inner membrane (IM) are designated by dashed lines. (B) A magnified view of one subunit of an RND-type inner membrane pump. Periplasmic subunits are designated DC (yellow), DN (red), PN1 (light blue), PN2 (purple), PC1 (orange), and PC2 (green). The transmembrane (TM) and membrane-associated helices are designated blue. RND, resistance–nodulation–cell division.

that occupy about a third of the length of the periplasmic space (Figure 1B). Its main function is to capture substrates (antibiotics such as tetracyclines, macrolides, and aminoglycosides) from the outer leaflet of the inner membrane or periplasm (or both) and transport them to the OMP for export, with the proton motive force the energy provider to facilitate substrate translocation^{25,26}. A subset of these RND-type inner membrane pumps is also responsible for the transport of lipids to the outer membrane to promote membrane biosynthesis and stability. Therefore, the development of compounds that inhibit these critical transporters represents a novel and potent strategy to combat MDR, XDR, and TDR bacterial infections.

Technical and technological advances for membrane structural biology

To date, most of the structural and functional information obtained regarding the RND transporters has been from X-ray crystallographic and biochemical studies. Our knowledge of the three-dimensional (3D) molecular orientation, assembly states, and transport mechanisms of this transporter superfamily has greatly advanced since the first published structure of AcrB, the IMP from the AcrAB-TolC efflux system²⁷, almost 20 years ago. Since then, the structures of several similar yet distinct RND IMPs have been elucidated (Table 1). These data have substantiated the importance of the IMPs and have provided valuable information on the binding and transport of their substrates. Solved IMP structures, in combination with large *in silico* chemical libraries, allow docking tools such as AutoDock Vina²⁸, Schrödinger Glide^{29,30}, and UCSF Dock³¹ to effectively identify and assess binding capabilities of potential inhibitory compounds. One limitation, however, of using X-ray crystallography for 3D protein reconstruction is that the method requires the production of a static crystal lattice, providing only a singular snapshot of these IMPs, forcing us to piece

together mechanistic details of substrate transport. To help overcome this, molecular dynamics simulations such as protein in atomistic details coupled with coarse-grained environment (PACE) have been employed. This technique was successful in modeling conformational changes of AcrB (the critical IMP from *E. coli*) upon indole transport and may be an effective tool to model the transport mechanisms of IMP inhibitors³².

Despite these efforts, the difficulty of producing high-quality crystals suitable for structural determination of membrane proteins remains a significant challenge. To solve these structures via crystallography, investigators typically need to start with a high concentration of mostly pure protein³³. Many times, the concentrations of isolated membrane proteins from bacterial or eukaryotic expression systems are insufficient to produce high-quality well-ordered crystals, while solubilization of the cell membrane for protein extraction often leads to impurities that significantly hinder the crystallization process³⁴. Historically, a detergent-based buffer has been used to facilitate removal of membrane proteins from their cellular membrane environment. This is not a trivial step, as both the type and concentration of detergent need to be experimentally determined with the overall goal to extract from the membrane a soluble protein in its functional state. Often, this trial-and-error method is unsuccessful. Additionally, detergent-based extraction eliminates the native lipid bilayer that may be required to maintain the protein in its native conformation³⁴. As an alternative, lipid bilayer mimetics such as bicelles³⁵, lipid cubic phase³⁶ and nanodiscs derived from native cell membrane nanoparticle systems (NCMNS)³⁷, and styrene maleic-acid lipid particles (SMALPs)³⁸ are being developed and successfully employed to help determine protein structures in their native lipid-associated state. These detergent-free systems may be beneficial for the continued study of RND transporters not only with X-ray

Table 1. Solved structures of the inner membrane protein component of the RND-type of bacterial efflux system.

Organism	Inner membrane protein	Year	Protein Data Bank ID	Method
<i>Escherichia coli</i>	AcrB	2002	1IWG ²⁷	X-ray
		2006	2DHH ³⁹	X-ray
<i>Pseudomonas aeruginosa</i>	MexB	2009	2V50 ⁴⁰	X-ray
<i>E. coli</i>	CusA	2010	3K07 ⁴¹ , 3KSS ⁴¹ , 3KSO ⁴¹	X-ray
<i>Cupriavidus metallidurans</i>	ZneA	2013	4K0E ⁴² , 4K0J ⁴²	X-ray
<i>Neisseria gonorrhoeae</i>	MtrD	2014	4MT1 ⁴³	X-ray
		2020	6VKS ⁴⁴ , 6VKT ⁴⁴	Cryo-EM
<i>Campylobacter jejuni</i>	CmeB	2017	5LQ3 ⁴⁵ , 5T0O ⁴⁵	X-ray
<i>Burkholderia multivorans</i>	HpnN	2017	5KHN ⁴⁶ , 5KHS ⁴⁶	X-ray
<i>Acinetobacter baumannii</i>	AdeB	2019	6OWS ⁴⁷	Cryo-EM
<i>Mycobacterium smegmatis</i>	MmpL3	2019	6OR2 ⁴⁸	X-ray
		2019	6AJF ⁴⁹	X-ray

Cryo-EM, cryo-electron microscopy; RND, resistance–nodulation–cell division.

crystallography but also with additional methods such as cryo-electron microscopy (cryo-EM)⁵⁰, X-ray free-electron laser (XFEL)⁵¹, and other biophysical techniques³⁴.

Emergence of cryo-electron microscopy as a powerful tool to study membrane complexes

X-ray structures essentially portray a protein in a rigid conformation, bound by the energetics and constraints of the crystal lattice. These constraints can introduce structural artifacts, particularly for protein–ligand interactions and for the spatial orientation of multi-protein assemblies⁵². Owing to these and other limitations, how and where substrates bind the RND IMPs and how complete RND transporter systems assemble can be difficult to structurally observe solely on the basis of X-ray crystallography. The emergence of single-particle cryo-EM has provided us with a promising alternative and allows many of the limitations of X-ray crystallography to be surpassed. Improved electron detectors and image processors have empowered cryo-EM to develop from a technique that provides “blob-like” low-resolution structures to the ability to compete with X-ray crystallography by solving high-resolution structures of large protein complexes in their native conformation^{53,54}. In single-particle cryo-EM, images of individual proteins or protein complexes are collected and processed to generate 3D reconstructions⁵⁵. There are at least two major advantages: (1) The amount of protein required for cryo-EM is substantially less than that for X-ray crystallography. This eliminates many of the problems often encountered when trying to produce large quantities of recombinant protein. (2) Cryo-EM has the ability to visualize more than one conformational state in a sample. Since proteins and protein complexes greater than 100 kDa are well suited for this technique, this makes RND transporters ideal protein targets for this method. This is highlighted by the seminal work detailing the structural assembly of the *E. coli* AcrAB-TolC⁵⁶ and the *Pseudomonas aeruginosa* MexAB-OprM⁵⁷ tripartite transport systems by cryo-EM. Cryo-EM also has the potential to detect substrates bound at multiple locations during the transport process, through the capture of intermediate states. Indeed, we recently used cryo-EM to solve the structure of the *A. baumannii* IMP component of the AdeABC efflux transporter, AdeB⁴⁷.

The ability to solve protein–antibiotic complexes

Through cryo-EM, we also determined two structures of the gonococcal MtrD transporter. We were able to elucidate how this multidrug efflux transporter specifically interacts with substrates in a more straightforward manner as we did not need to optimize crystallization conditions suitable for crystallizing MtrD bound with drugs. In *Neisseria gonorrhoeae*, the causative agent of the sexually transmitted infection gonorrhea, the multiple transferable resistance efflux system, MtrCDE, exports a wide variety of diverse antimicrobial agents from the cell, and its expression is a major contributor to β -lactam and macrolide resistance⁵⁸. The inner membrane efflux transporter, MtrD, is responsible for the recognition and transport of substrates in concert with the periplasmic MFP MtrC and OMP MtrE. Therefore, targeting MtrD is a viable strategy to increase the potency of antibiotics in order to eliminate

gonococcal infection. Cryo-EM was used to successfully determine structures of the MtrD efflux transporter, carrying a mosaic-like sequence, in the presence of bound antibiotics⁴⁴. These structures enabled us to identify important residues for drug recognition, and several of these residues were independently verified *in vivo*⁵⁹, as well as modes of MtrD–drug interactions. The drug molecules were found to bind deeply at the distal drug-binding site in the periplasmic domain of MtrD (Figure 2A). Important residues that stabilize antibiotic–MtrD binding were identified (Figure 2B) and a mechanism of substrate transport was able to be postulated. It is likely that antibiotics enter the channel from the periplasmic cleft created by domains PC1 and PC2, then sequentially bind the proximal and distal binding sites. These binding sites guide substrate

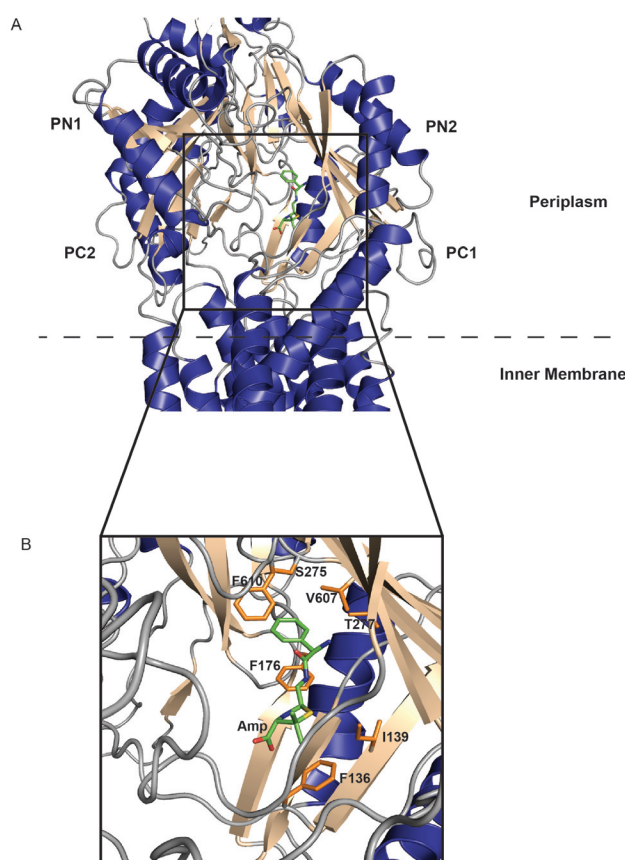


Figure 2. Antibiotic-bound cryo-EM structure of the *Neisseria gonorrhoeae* RND-type inner membrane pump, MtrD (adapted from Protein Data Bank ID 6VK5). (A) α -helices (blue), β -sheets (wheat), and loops (gray) depict the overall secondary structure of MtrD. A hydrolyzed, decarboxylated ampicillin molecule (green) is bound deep within the cavity formed by the orientation of the periplasmic domains PC1, PC2, PN1, and PN2. The inner membrane–periplasm lipid boundary is represented by a dashed line. (B) A magnified view of the ampicillin-binding region. Important amino acid side chains involved in substrate recognition/stabilization are shown in orange. Amp, hydrolyzed, decarboxylated ampicillin; cryo-EM, cryo-electron microscopy; RND, resistance–nodulation–cell division.

movement through the channel and the eventual release to the OMP for export from the cell. Through this cryo-EM structural approach coupled with genetic studies, we also identified that the conserved charged amino acids R714 and E823 are critical for the recognition of macrolides and provide clinical non-susceptibility to azithromycin (azithromycin and ceftriaxone are recommended as the first choice for dual treatment of gonorrhea⁶⁰). Interestingly, a global meta-analytical approach was used to analyze 4,852 clinical isolates. The study found that mutations at positions 714 and 823 of MtrD in these isolates led to azithromycin resistance well above the Clinical and Laboratory Standards Institute (CLSI) azithromycin non-susceptibility threshold⁶¹, confirming our hypothesis that amino acid changes at MtrD positions 714 and 823 could lead to clinically significant levels of azithromycin non-susceptibility resistance. Because of the high quality of the structural data, we were also able to detail the proton transfer process within the proton relay network, which provides the proton motive force to power up this multidrug efflux transporter. Taken together, these structural studies allowed the correlation of spontaneous resistance mutations within MtrD to specific locations within the protein and can aid in the design of new and more effective inhibitory compounds to obstruct the principal multidrug efflux mechanism in *N. gonorrhoeae*.

Cryo-electron microscopy as a tool to study the membrane proteome

The purification of membrane proteins to near homogeneity can be a difficult and laborious process. This is especially challenging when trying to express eukaryotic membrane proteins using either current cell culture systems or non-eukaryotic

expression methods. These purifications can lead to dilute, impure samples. Although low concentration and purity are not necessarily detrimental for reconstructing small (<500 kDa) water-soluble protein complexes⁶², they are often roadblocks for obtaining high-quality crystals of larger complexes, especially those that associate with the membrane. In many cases, cryo-EM is able to help overcome these homogeneity and purity problems. After data collection and initial image processing, *in silico* purification of the particle set allows separation of particle classes based on the results of 2D and 3D *ab initio* classifications. This process helps to separate different views of the target protein from those that belong to impurities, which allows map building of the protein of interest. While methods have been developed to build maps of multiple water-soluble protein complexes from a heterogeneous, partially purified sample⁶³⁻⁶⁹, we are currently developing an iterative methodology to handle impure, heterogeneous samples for structural determination of RND transporters and other membrane-bound complexes by cryo-EM. To test our strategy, we determined cryo-EM structures of the *A. baumannii* 70S ribosome⁷⁰. The intact 70S complex as well as the individual 30S and 50S subunits were able to be identified in distinct 2D class averages from a single cryogenic sample (Figure 3A). Using 3D variability analysis, we were able to detect the inter-particle motions of the 70S ribosome in different tRNA-bound states (Figure 3B), as well as visualize the ribosome in various conformations upon the introduction of ribosomal-specific substrates, thus allowing an enhanced understanding of the structural dynamics of ribosomal function from one sample. These techniques can be effectively extended to analyze membrane isolates. Indeed, with cryo-EM, it will soon be possible to

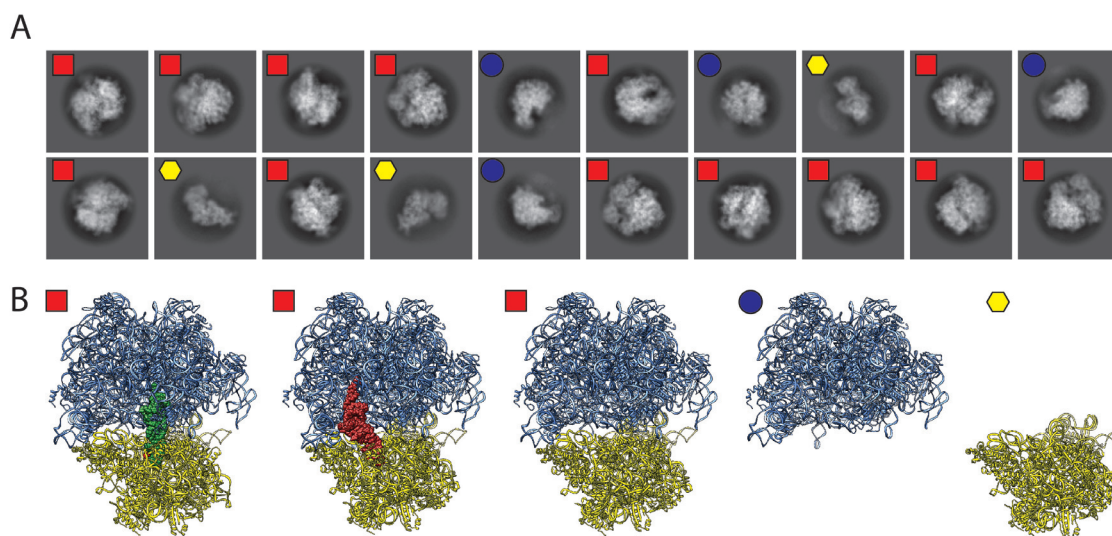


Figure 3. Ribosomal structures determined by single particle cryo-EM. (A) Two-dimensional class averages obtained from a single cryo-EM experiment. Ribosomes were isolated from the *Acinetobacter baumannii* bacterium and flash-frozen onto a cryogenic grid. Shown are three separate classes that differentiate the 70S complex (red squares) from the individual 50S (blue circles) and the 30S (yellow hexagons) subunits. (B) Further computational sorting and analysis revealed three separate states of the intact 70S ribosome: tRNA bound at the P-site (green spheres), the E-site (red spheres), or empty. cryo-EM, cryo-electron microscopy.

simultaneously solve structures of several bacterial membrane proteins to near atomic resolution⁶⁷. Non-homogeneous sample preparations will no longer be restrictive for these membrane isolates, and significant data can be obtained from a single, heterogeneous protein sample.

In addition to the generation of high-quality structures of protein–substrate complexes that are challenging to solve with X-ray crystallography, cryo-EM has the potential to be used in a broader scope as a conduit to structural systems proteomics. Many cellular machineries consist of protein complexes and enzymatic pathways that span both the inner and outer membranes of Gram-negative bacteria as well as eukaryotic mitochondria. With the advancements of cryo-EM as well as the recent development of native mass spectrometry⁷¹, it is now possible to study these proteins within intact membranes. It is not unrealistic to foresee how multiple structures of a complex or enzymatic chain can be solved simultaneously with the help of these cutting-edge technologies. This will enable the identification of important protein–protein contacts and help discern how these proteins assemble into a functional complex. The end result is the development of an integrated systems approach to structural biology that will greatly aid in elucidating the membrane proteome. This, in turn, will significantly advance our knowledge of how cells develop multidrug resistance and shed light on new membrane complexes and transport systems as potential targets for the development of new inhibitors or biocides or both.

Future prospective

The field of structural biology has developed into a powerful tool in the fight against drug-resistant bacterial infections. X-ray crystallography has been the standard for protein structural determination for decades and the foundation on which structure-based drug design has been built. With the advancement of additional techniques, such as cryo-EM, cryo-electron tomography (cryo-ET)⁷², microcrystal electron diffraction (micro-ED)⁷³, and XFEL⁵¹, the ability to generate structural data has never been greater. Protein structures in their native state as well as structures of protein–ligand and protein–protein complexes are becoming easier to determine. It is also possible to thoroughly analyze the dynamics of biomacromolecular

systems via these state-of-the-art technologies. This is especially beneficial for the study of bacterial efflux systems, whose inhibition holds great promise as a method to increase the potency of current antibiotic treatment regimens.













Through cryo-EM, it is possible for the IMPs in *E. coli*, *N. gonorrhoeae*, *A. baumannii*, and *Campylobacter jejuni*, among others, to be determined in their solution state, with and without bound substrate. Combined, these structures will allow a more complete description of how these pumps recognize and transport substrates. The attractiveness of these IMPs as potential drug targets is highlighted in a recent study that identified 42 new efflux pump inhibitors from a screen of about 50,000 diverse compounds⁷⁴. The ability to determine how and where these substrates interact within the pumps would greatly enhance the optimization efforts required to produce more potent inhibitors, as these compounds have been shown to block the substrate transport pathway at multiple locations. This example highlights the ability of structural biology to be a cornerstone for cooperative scientific techniques used in conjunction for inhibitor design and drug development.

Although there has been significant success in solving individual components of the RND transporter superfamily, it has been a formidable task to obtain structural information of a completely assembled tripartite efflux complex, which spans the entire bacterial cell envelope. The most successful visualizations have been for the AcrAB-TolC efflux system⁵⁶ by cryo-EM and its *in situ* structure using cryo-ET⁷⁵. Through the utility of these powerful techniques combined with optimized sample preparations, it is likely that additional complete transport machineries (Figure 1) will be able to be viewed as a complete complex as opposed to each component solved separately. This improved methodology will extend to other important biological systems such as electron transport chains, respiratory complexes, and toxins^{76–78}. The intimate understanding of these transporters, porins, channels, and other proteins that make up the membrane proteome will greatly enhance the ability to identify new targets and design novel compounds to combat the rapid evolution of MDR strains of bacteria.

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